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(54) Title: USE OF RECEPTOR AGONISTS TO STIMULATE SUPEROXIDE DISMUTASE ACTIVITY (57) Abstract The present invention relates to the use of a substance for the manufacture of a composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells. In particular, the invention relates to the use of a substance for the manufacture of a composition for prophylaxis or treatment of a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical. Further, the invention relates to a method for determining the effect of a substance with respect to stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells and to substances which have been selected by said method. Within the scope of the invention is a method of preventing, diminishing, controlling or inhibiting a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical in a patient who has been established to have a high risk of developing a such disease or disorder, or who has developed a such disease or disorder, the method comprising administering an effective amount of a substance which is capable of stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells.		

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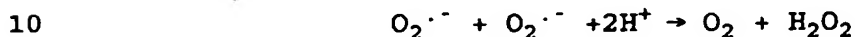
USE OF RECEPTOR AGONISTS TO STIMULATE SUPEROXIDE DISMUTASE ACTIVITY

FIELD OF INVENTION

The present invention relates in particular to the use of a substance for the manufacture of a composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD.

GENERAL BACKGROUND

Superoxide dismutases (SOD) protect against superoxide radical by catalysing its dismutation.



There are two intracellular SOD's, the cytosolic copper and zinc containing superoxide dismutase (CuZn-SOD) and the mitochondrial matrix manganese containing superoxide dismutase (Mn-SOD). The extracellular space contains the extracellular superoxide dismutase (EC-SOD). EC-SOD is a tetrameric, Cu and Zn-containing, glycoprotein (Marklund, 1982) which is synthesized and secreted by only a few cell-types including fibroblasts, and some glia cells (Marklund, 1990) and smooth muscle cells (this application). EC-SOD has a high affinity for heparan sulfate and exists anchored to heparan sulfate proteoglycans in the glycocalyx of cell surfaces and in the tissue interstitial matrix (Karlsson et al., 1988; Marklund and Karlsson, 1989; Sandström et al., 1993; Karlsson et al., 1994).

25 The effects of the superoxide radical and derived products on blood vessel functions have attracted increasing attention. Direct damaging effects on the endothelium (Mehta et al., 1989) and other vessel components, involvement in the oxidation of LDL (Steinbrecher et al., 1990; Heinecke et al., 1986; Kawamura et al., 1994) and hence potential involvement in atherosclerosis, noxious interactions with •NO (Beckman

et al., 1990; Huie et al., 1993; Ischiropoulos et al., 1992; Koppenol et al., 1992; Darley-Usmar et al., 1992), and direct effects on vessel tonus have been reported (Katusic and Vanhoutte, 1989). However, despite the potential importance of the superoxide radical, little is known about the endogenous protection of the vessel wall provided by superoxide dismutases.

DETAILED DESCRIPTION OF THE INVENTION

In order to assess the role of SOD in blood vessel homeostasis the contents of the secretory, interstitial EC-SOD (Marklund, 1982), the cytosolic CuZn-SOD (McCord and Fridovich, 1969) and the mitochondrial matrix Mn-SOD (Weisiger and Fridovich, 1973) was determined in human coronary artery, aorta and saphenous vein. Comparatively little CuZn-SOD and Mn-SOD, but exceptionally large amounts of EC-SOD were found.

However, the cell contents of vascular walls are low, thus comparison with other tissues on a DNA content basis indicates intermediate to high average contents of both CuZn-SOD and Mn-SOD of cells in the vascular wall. Analysis of EC-SOD by immunohistochemistry indicates an even distribution in the vessel wall, including large amounts in the arterial intima. The EC-SOD concentration in the human arterial wall interstitium is high enough to prevent most putative pathophysiological effects of superoxide radicals, such as oxidation of LDL, with the exception of the formation of the deleterious peroxynitrite under maximal rates of nitric oxide synthesis.

EC-SOD exists in the interstitium of all tissues and as shown in Table 1 particularly large amounts are found in man in the wall of arteries, bronchi (unpublished), skin (unpublished), uterus, thyroid gland and lung (Marklund, 1984). The carboxy-terminal heparan-sulfate-binding domain (Sandström et al., 1992) is highly susceptible to proteolytic truncation (Karls-son et al., 1993), such cleavage results in rapid loss from

the tissue interstitium and may be caused by proteolytic enzymes released/activated in the inflammatory response.

Of all human tissues, the arterial wall interstitium contains by far most EC-SOD, Table 2. The enzyme is evenly distributed
5 over the wall, Fig 1, including large amounts in the intimal layer. Assuming a distribution volume of 30% of the arterial wall, an average EC-SOD activity of 15000-20000 U/ml in the interstitium can be calculated. This corresponds to about 110 μ g/ml of CuZn-SOD. Such concentrations are reported to
10 strongly suppress a variety of pathophysiological effects of the superoxide radical in *in vitro* models. The functions of EC-SOD in the wall should be to protect against deleterious effects of superoxide radical. Sources of the radical in the vessels could be activated neutrophil leucocytes, monocytes,
15 macrophages and other phagocytic leucocytes. There is also evidence for release by endothelial cells, smooth muscle cells, by not always well-defined mechanisms. There is evidence for formation by autoxidation of thiols (Heinecke et al., 1987). Superoxide can also be released as a byproduct
20 during synthesis of prostaglandins and leukotrienes (Kukreja et al., 1986).

There are two areas (partly interrelated) in which EC-SOD should fulfil particularly important role in the arterial interstitium.

25 1. LDL oxidation has been suggested to be a primary step in atherogenesis (Steinberg et al., 1989). After oxidation the LDL particles are taken up into macrophages through the scavenger receptor resulting in the eventual formation of foam cells. These reactions take place in the arterial intima, which is the site of formation of the atherosclerotic
30 plaque. In a variety of studies addition of SOD has been shown to retard the LDL oxidation (Steinbrecher et al., 1990; Heinecke et al., 1986; Kawamura et al., 1994) suggesting involvement of the superoxide radical in the process. Sup-

pression of such reactions should be a primary physiological function of EC-SOD.

2. $\cdot\text{NO}$ produced by the endothelium is a major physiological vasodilator (Palmer et al., 1987; Ignarro et al., 1987) and
5 also reduces adhesion of platelets (Radomski et al., 1987) and leukocytes (Gaboury et al., 1993) to the vascular wall. $\cdot\text{NO}$ may also be formed by activated phagocytic cells, and its synthesis can be induced to occur in smooth muscle cells (Beasley et al., 1991). $\cdot\text{NO}$ reacts with superoxide at a
10 nearly diffusion-limited rate to form the very toxic species, peroxynitrite (Beckman et al., 1990; Huie et al., 1993). Peroxynitrite, in itself oxidizing, may nitrate proteins (Ischiropoulos et al., 1992), may induce LDL oxidation (Darley-Usmar et al., 1992), and may decompose to other strongly
15 oxidizing species (Koppenol et al., 1992). Thus, the interaction between superoxide and $\cdot\text{NO}$ both reduces the physiological effects of $\cdot\text{NO}$ and leads to the formation of toxic compounds. The *in vivo* occurrence of peroxynitrite is indicated by the extensive nitration of protein tyrosines found
20 in human atherosclerotic lesions (Beckman et al., 1994). The $\cdot\text{NO}$ concentration in isolated rabbit aorta, with formation by the endothelium maximally stimulated, has been measured to be about $0.85 \mu\text{M}$ (Malinski et al., 1993), although the concentration *in vivo* may be lower due to scavenging of $\cdot\text{NO}$ by
25 hemoglobin (Lancaster, 1994). Using the high rate constant for peroxynitrite formation, $6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Huie et al., 1993), it can be calculated that about 19000 U/ml (EC-)SOD is needed to compete equally with $0.85 \mu\text{M}$ $\cdot\text{NO}$ for superoxide radicals occurring in the vascular wall. The high concentra-
30 tions of EC-SOD found in the human arterial wall interstitium should be sufficient to compete with basal concentrations of $\cdot\text{NO}$ encountered in the vascular wall, but may allow significant formation of peroxynitrite from superoxide under maximum agonist stimulation and when $\cdot\text{NO}$ is formed by inflammatory
35 cells and by smooth muscle cells after nitric oxide synthase induction. Although a variety of sources have been suggested, the rate of endogenous formation of superoxide radical (and

thus potentially of peroxynitrite) in the vascular wall is not known. Under some pathophysiological circumstances, however, the rate of superoxide formation must approach that of nitric oxide, as indicated by the results of administration of large amounts of SOD. Thus injection of a heparan sulfate-binding SOD derivative reduced the blood pressure in spontaneously hypertensive rats (Nakazono et al., 1991); addition of SOD enhanced acetylcholine-induced relaxation of aortas from diabetic rats (Hattori et al., 1991); in vivo administration of liposome-encapsulated CuZn-SOD enhanced the response to acetylcholine of isolated aortic rings derived from cholesterol-fed rabbits (White et al., 1994); and finally, transgenic overexpression of human EC-SOD reduced cold-induced vasogenic brain edema in mice (Oury et al., 1993). All these studies were made in species with considerably less arterial wall EC-SOD than is found in man, Tables 2 and 3, and the results emphasize the potential protective role of the high EC-SOD level of the human arterial wall interstitium.

Thus, although the EC-SOD content of the arterial intimas is very high, it may under high nitric oxide synthesis allow significant formation of deleterious peroxynitrite, or conversely when there is increased superoxide formation be barely sufficient to preserve nitric oxide for its physiological functions such as vasodilation (reduction of blood pressure) and deactivation of platelets (anticoagulant effect) and phagocytic leukocytes (antiinflammatory effect). The extensive nitration of protein tyrosines in human atherosclerotic plaques (Beckman et al., 1994) indicates that the high EC-SOD content of human arteries may be insufficient under pathological conditions.

Changes in arterial EC-SOD content below or above the average content observed, Table 2, may thus significantly alter various pathological processes in the blood vessel wall.

There exists a common mutation in human EC-SOD, Arg213Gly, exchanging the positively charged Arg by Gly in the heparin binding domain of EC-SOD (Sandström et al., 1994). This results in reduced affinity for heparin and heparan sulfate.

5 Individuals carrying this mutation show an, in average, 8-fold increased plasma EC-SOD activity. This phenotype occurs in a few percent of the population in Sweden (Sandström et al., 1994), Japan (Adachi, T., Nilsson, P., Marklund, S.L., unpublished) and the USA (Folz et al., 1994). Remarkably, the

10 phenotype has been shown to be caused by the *same nucleotide exchange* in all investigated individuals in all three populations. It has previously been shown that an EC-SOD truncation mutant (Sandström et al., 1992), with a heparin affinity reduced to the same extent as that of homotetrameric Arg213Gly-

15 EC-SOD, is lost from the tissue interstitium 4-fold more rapidly than wild-type EC-SOD (but still 3-fold less rapidly than EC-SOD lacking heparin affinity) (Karlsson et al., 1994). This suggests that EC-SOD in the heterozygotes is more rapidly lost from tissues reaching and accumulating in the

20 vasculature via capillary membranes and the lymph flow. The result is the 8-fold increased plasma activity and also an increased activity on and beneath the endothelium (Sandström et al., 1994), as can be revealed by injection of heparin. On balance the activity in the tissue interstitium should be

25 decreased.

The remarkable common occurrence of the same mutation in the three different populations suggests that it confers some advantage, e.g. regarding vascular disease, possibly balanced by some disadvantage regarding disease in other organs. To

30 evaluate this notion, the exact frequency of the mutation in Västerbotten County (where Umeå is situated) was established by screening plasma from 4498 persons participating in the randomized WHO MONICA population investigation, and it was found that 3.65% of the population showed this phenotype.

35 Carriers of the phenotype did not differ from non-carriers with regard to conventional risk factors for atherosclerosis such as blood pressure, blood lipids, and smoking habits.

Blood was also collected from patients admitted to the Umeå University Hospital for stroke (n=801), and among these the phenotype frequency was 1.00%. This calculates to an odds ratio for stroke among carriers of the mutation of 0.267, confidence interval 0.11-0.54. Preliminary studies among patients with coronary heart disease point in the same direction (Schampi, I., Israelsson, K., Peltonen, M., Nilsson, P., Marklund, S.L., Asplund, K., in preparation). There is thus a highly significant decreased risk of atherosclerosis-related disease in individuals carrying the mutation. This finding suggests that there is a large therapeutic potential in long-term induction of EC-SOD synthesis in the blood vessels by small molecules.

A common problem after percutaneous transluminal coronary angioplasty (PTCA) is both early vasospasm as well as late proliferative response leading to recurrent constriction of the lumen, apparently as a response to trauma. It has been shown that there is an early enhanced formation of superoxide radicals in the vascular wall (Laurindo et al., 1991). This suggests that administration of EC-SOD into the vascular wall during dilation or treatment by use of small molecules that enhance EC-SOD synthesis might attenuate sequelae after PTCA.

It has previously been shown that only few cell types secrete EC-SOD and that fibroblasts and glia cells may be important sources in the body (Marklund, 1990). The main source of EC-SOD in the arterial wall is apparently the smooth muscle cells which secrete large amounts of EC-SOD as measured by ELISA, Fig 1. EC-SOD synthesis by fibroblast has previously been shown to be influenced by inflammatory cytokines, IFN- γ induces whereas TNF α , IL-1 α and particularly TGF β repress the synthesis (Marklund, 1992). On the other hand, it was not possible to show any specific effect on synthesis of a variety of types of oxidant stress (Strålin and Marklund, 1994).

A variety of factors have now been tested for effect on human arterial smooth muscle cells (SMC) EC-SOD synthesis, Table 4. It has been found that the SMC's respond (essentially like fibroblasts) to the inflammatory cytokines mentioned above, but also to IL-4 and IL-8. Novel findings are also distinct responses to various growth factors. The most particular novel finding is the marked response to a long series of vasoactive factors, and e.g. the activator of protein kinase C, phorbol 12-myristate 13-acetate (PMA). Also fibroblasts were found to respond to PMA. The remarkably wide variety of factors that influence EC-SOD synthesis suggest multiple mechanisms for cellular control of EC-SOD synthesis. A variety of other natural and synthetic compounds might, after identification by screening (Example 2), be used for control of EC-SOD synthesis.

It is thus apparent that it should be possible to influence the EC-SOD content of the arterial wall and other tissues in vivo with the various factors listed in Table 4. Since the SMC's respond to such a variety of vasoactive factors, it is highly likely that they should respond to other vasoactive factors which have not yet been tested. They should also respond to a variety of synthetic and natural drugs influencing smooth muscle cells by the same receptor mechanism as those used by the factors of Table 4. Already existing drugs or new to be developed could be used to influence (increase) arterial wall EC-SOD content. Results could be improved blood pressure control, reduction of inflammation and suppression of formation of atherosclerotic lesions.

There is a large body of evidence implicating superoxide and other oxygen free radicals in diabetes and diabetes complications. Thus, glucose, at concentrations relevant to diabetes, autoxidizes under formation of oxygen free radicals (Hunt et al., 1988). Glucose glycates amino groups in proteins (amino terminal, lysine). Measurement of protein glycation (mostly hemoglobin) is routinely used in management of diabetes patients, and has proved to correlate strongly with complica-

tions such as retinopathy and nephropathy. Glycated proteins autoxidize easily (much more so than glucose itself) under formation of superoxide radical (Arai et al., 1987; Mullarkey et al., 1990; Sakurai and Tsuchiya, 1988). The autoxidation leads to protein fragmentation, DNA nicking, etc. Ascorbate in plasma is reduced and dehydroascorbate increased in diabetic patients, probably due to oxidation by oxygen free radicals (Hunt et al., 1992). Superoxide efficiently oxidizes ascorbate. Many of the diabetes complications are related to enhanced atherosclerosis. Incubation of LDL with concentrations of glucose relevant in diabetes leads to peroxidation, apparently a primary step in atherogenesis. SOD inhibits this process (Hunt et al., 1990; Kawamura et al., 1994). Incubation of endothelial cells with glycated proteins leads to oxidative stress, e.g. peroxidation, SOD inhibits (Yan et al., 1994). Aortas from animals with e.g. streptozotocin-induced diabetes show reduced relaxation induced by acetylcholine and other agonists inducing NO-release. SOD normalizes the response showing that the effect is due to strongly increased formation of superoxide in the diabetic vessel wall (Pieper et al., 1992; Tesfamariam and Cohen, 1992; Hattori et al., 1991). The same situation can be induced by incubation of aortas with high glucose concentration (Tesfamariam and Cohen, 1992). Addition of SOD to the medium has been shown to suppress peroxidation of LDL in a variety of *in vitro* cell culture systems, indicating that the superoxide radical may be involved in LDL oxidation *in vivo* and hence in atherogenesis. The blood flow and conduction velocity in peripheral nerves is reduced in diabetes, and this is suggested to be a major cause of diabetes polyneuropathy. In animals, these abnormalities have been shown to be reduced by transition metal ion chelators, which presumably reduced formation of oxygen radicals (Cameron and Cotter, 1995). Injection of glycated albumin induced glomerulopathy in rats. Antibodies raised against that albumin prevented this diabetes-mimicking kidney disorder (antioxidants were not tested in the model) (Cohen et al., 1995). There is increased superoxide formation in renal arterioles resulting in reduced responses to nitric

oxide. SOD restores the responses (Ohishi and Carmines, 1995). The (cytosolic) CuZnSOD is reduced in tissues in diabetes. This is at least partially due to glycation-induced fragmentation of the protein (Arai et al., 1987). EC-SOD is not inhibited by glycation (but suffers reduced affinity for heparan sulfate because of lysine glycation). The pancreatic B-cells show exceptional susceptibility to superoxide, e.g. formed by alloxan (Grankvist et al., 1979; Grankvist et al., 1981), and injection of polyethylene-substituted SOD has been shown to suppress insulinitis in NOD mice (Horio et al., 1994). This suggests that SOD might halt progression of loss of β -cell function in diabetes.

Collectively these studies suggest that increased superoxide dismutase activity might ameliorate diabetes complications. Since it is a chronic disease, enhanced EC-SOD activity should preferably be accomplished by small molecules enhancing EC-SOD synthesis. Enhancement of synthesis in several cell types, e.g. smooth muscle cells, fibroblasts and glia cells, may be advantageous.

It has also been found that human bronchi contain large amounts of EC-SOD. The source in the bronchi are likely, as in the blood vessels, the smooth muscle cells. These cells are likely to respond like the SMC's of the vessels to similar substances and to factors specially influencing bronchial reactions such as tonus. NO is known to be formed in bronchi and to exert a dilating function. Peroxynitrite could like in blood vessels be formed, particularly in inflammatory states.

The use of drugs enhancing bronchial EC-SOD synthesis is thus envisaged for the treatment of bronchial diseases involving inflammation and constriction, such as asthma.

In human skin the EC-SOD content is high and also accounts for an unusually large proportion of the total SOD activity, Table 1. Variation of the EC-SOD content may influence diseases in the skin, especially when inflammation is involved.

Enhanced EC-SOD may also be important for wound healing. Superoxide radicals are formed by activated phagocytis leukocytes and e.g. as a by-product upon synthesis of prostaglandins and leukotrienes (Kukreja et al., 1986). Fibroblasts are
5 a likely source of EC-SOD in the skin, but synthesis by other cell types is conceivable. The basic idea here is to increase (or decrease) the synthesis of EC-SOD in skin by various effector molecules, especially among the inflammatory cytokines, growth factors, steroids, prostaglandins, phorbol
10 esters, protein kinase C activators, etc.

The uterus also contains large amounts of EC-SOD, Table 1. The source in uterus should be the smooth muscle cells of the myometrium. They should respond in a similar manner as the blood vessel smooth muscle cells to factors described below.
15 Given the hormonal control of uterus, sexual hormones should also influence EC-SOD synthesis.

The idea here is to influence pathology induced by superoxide radicals by influencing the level of EC-SOD synthesis. Superoxide radicals may exert direct effect on myometrial
20 contractility (Katusic and Vanhoutte, 1989). NO is apparently synthesized in the myometrium since nitric oxide synthase can be found in the myometrium. NO exerts a relaxing effect on the smooth muscle cells. Since NO occurs, the toxic peroxynitrite can also be formed in the myometrium. EC-SOD would
25 thus enhance relaxing effects of NO, prevent direct effects on the myometrium, and reduce formation of the toxic peroxy-nitrite.

It may be important to regulate EC-SOD in diseases involving inflammation, and in conditions with enhanced or reduced contractility during parturition.
30

There is considerable evidence linking inflammatory damage to superoxide and other oxygen free radicals. Thus, neutrophil leukocytes (Babior et al., 1973), monocytes (Johnston et al., 1974), macrophages (Johnston et al., 1978) and eosinophil

leukocytes (de Chatelet et al., 1977) secrete large amounts of superoxide upon activation. The superoxide and secondary products formed from it seem to be of great importance for the bacteriocidal and cytotoxic actions of the cells. Other
5 cell types can also be stimulated to release superoxide although the amount is smaller than that of the granulocytes; endothelial cells (Gryglewsky et al., 1986; Laurindo et al., 1994), fibroblasts (Meier et al., 1989), smooth muscle cells mesangial cells (Radeke et al., 1990), B-lymphocytes (Maly,
10 1990) and kidney tubulus cells (Rovin et al., 1990). Superoxide is also formed as a byproduct during prostaglandin and leukotriene (Kukreja et al., 1986) biosynthesis. Moreover, in a variety of situations administration of SOD has been shown to suppress inflammation (Vaille et al., 1989; Menander-
15 Huber, 1980; Dowling et al., 1993; Goebel and Storck, 1983).

Further indications are derived from studies in mice lacking EC-SOD. To study the function of EC-SOD *in vivo*, mice carrying a targeted disruption of the EC-SOD gene have been generated (Carlsson et al., 1995). EC-SOD null mutants were found
20 to develop normally and have remained healthy for over a year. Thus under normal pathogen-free animal caring, other systems such as ascorbate and ceruloplasmin may compensate for the loss of EC-SOD. However, when stressed by exposure to >99% oxygen, the null mutant mice displayed a considerable
25 reduction in survival time compared to wild type mice and an earlier onset of severe lung edema. Apparently the loss of EC-SOD exacerbates the inflammatory reaction induced by high oxygen tension. In preliminary studies, the EC-SOD null mice also display enhanced inflammatory response when exposed to
30 ozone, dextran sulfate in drinking water (results in a colitis resembling ulcerative colitis), and puncture of eardrums (enhanced inflammatory middle ear exsudate and delayed healing). Together the findings suggest an *in vivo* role of EC-SOD as modulator of inflammation.

35 The basic idea of this invention is to alter, e.g. increase, the level of EC-SOD in blood vessels, bronchi, lung, kidney,

skin, gut, uterus, cornea, joints, central nervous system, possibly other organs such as the heart by altering, e.g. enhancing, the endogenous synthesis of EC-SOD, using drugs as outlined above. It is evident that for some drugs, the level
5 may be altered in only one of the above-mentioned tissues whereas other drugs may alter the level in several or all of them. It may even be possible that the alteration is different in the different tissue, e.g. that in some tissues the level will be increased whereas in other tissues it is
10 decreased. Such situations are within the scope of the present invention as long as a overall beneficial effect is obtained.

Within the concept of this invention is also to decrease the level of EC-SOD in blood vessels, bronchi, lung, kidney,
15 skin, gut, uterus, cornea, joints, central nervous system, possibly other organs such as the heart if that should prove beneficial under some circumstances by decreasing the endogenous synthesis of EC-SOD, using drugs as outlined in the experimental part.

20 Within its broadest aspects, the present invention relates to the use of a substance for the manufacture of a composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells.

In a preferred embodiment, the cells are smooth muscle cells.
25 In one embodiment, the present invention relates to use according to the invention, wherein the substance exhibits agonist activity on a receptor selected from the group consisting of adenosin receptors, adrenoceptors, angiotensin receptors, atrial natriuretic peptide receptors, bradykinin
30 receptors, calcitonin gene-related peptide receptors, Ca^{++} channels, dopamine receptors, endothelin receptors, fibroblast growth factor, growth hormone, histamine receptors, 5-hydroxytryptamine receptors, interferon γ , interleukin-1, interleukin-4, interleukin-8, interleukin-10, interleukin-13,
35 leukotriene receptors, muscarinic receptors, neuropeptide Y

receptors, nitric oxide receptors, platelet derived growth factor receptors, prostanoid receptors, P₂ purinoceptors, 5-hydroxytryptamine receptors, tachykinin receptors, thrombin receptors, transforming growth factor β , tumor necrosis factor, vasopressin receptors, receptors for heparin and other sulfated glycosaminoglycans, epidermal growth factor receptors, protein kinase C modulators and insulin receptor..

In a preferred embodiment, the substance is a vasoactive factor. In the present specification and claims, a vasoactive factor is defined as a substance which has an effect on blood vessels, e.g. exhibits agonist activity on a vasoactive receptor. An agonist is defined as a molecule such as a drug, an enzyme activator, or a hormone, that enhances the activity of another molecule or receptor site whereas a receptor is a target site at the molecular level to which a substance becomes bound as a result of a specific interaction. In the present case, the site may be on the cell wall, on the cell membrane, or on an intracellular enzyme or another protein with regulatory function, and the substance bound may be a hormone or a drug where the binding interaction will trigger the release of EC-SOD or stimulate the synthesis of EC-SOD.

A full agonist is a ligand that binds to a receptor and causes a maximum biological response whereas a partial agonist is a ligand that binds to a receptor and causes a less than maximum biological response even when it occupies all of the available receptor sites. Both full and partial agonists are within the scope of the present invention.

By "stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells" is meant stimulating the transcription of the EC-SOD gene, stimulating the translation of EC-SOD messenger RNA or enhancing stability of mRNA, and/or stimulating the secretion of EC-SOD after protein synthesis from the cells.

There are a number of receptor systems which are involved in the regulation of the EC-SOD synthesis. Examples of potential such receptors as well as of agonists/antagonists of the specific receptor types are outlined in the following:

5 **Adenosine receptors:**

Nomenclature	A ₁	A _{2A}	A _{2B} A ₃
agonists	N ⁶ cyclopentyl-adenosine 2-Cl-N ⁶ cyclopentyl-adenosine	CGS21680 PAPA-APEC	APNEA
antagonists	DPCPX(8.3-9.3) 8-cyclopentyltheophylline(7.4)	CP66713(7.7) KF17837	1-ABOPX(8.1)

10

Adrenoceptors:

endogenous agonists: adrenaline, noradrenaline

Nomenclature	α_1	α_2
15 agonists	phenylephrine cirazoline	UK14304 BHT920
antagonists	WB4101(~9.2) prazosin(8.5-10.5)	prazosin(7.5) ARC239(8.0)

Nomenclature	β_1	β_2	β_3
agonists	noradrenaline	procaterol	BRL37344
20 antagonists	betaxolol(8.5) atenolol(7.0)	butaximine(6.2)	pindolol

Angiotensin receptors:

endogenous agonists: angiotensin 2, angiotensin 3

	Nomenclature	AT ₁	AT ₂
5	agonists		CGP42112
	antagonists	losartan(8.1)	PD123177

Atrial natriuretic peptide receptors:

10	Nomenclature	ANP _A	ANP _B
	agonists		CNP
	antagonists	[L- α -aminosuberic acid ^{7,23'}]- β -ANP(7.5)	

Bradykinin receptors:

15

 endogenous agonists: bradykinin, kallidin, T-kinin

	Nomenclature	B ₁	B ₂
	agonists	BK ₁₋₈	kallidin
	antagonists	[Leu ⁸]BK ₁₋₈ (6.7-7.3)	HOE40(8.6)

20

Dopamine receptors:

	Nomenclature	D ₁	D ₂
	agonists	SKF38393 fenoldopam	N0437 bromocriptine
5	antagonists	SCH23390	domperidone

Endothelin receptors:

endogenous agonists: endothelin 1, endothelin 2, endothelin 3

10	Nomenclature	ET _A	ET _B
	agonists		Sarfatoxin S6c
	antagonists	BQ123(7.4) FR139317(7.2)	[Cys ^{11,15}]ET-1

Histamine receptors:

15

	Nomenclature	H ₁	H ₂	H ₃
	agonists	2-(m-fluorophenyl)- histamine	dimaprit impromidine	imetit
	antagonists	triprolidine (9.9)	tiotidine(7.8)	thioperamide(8.4)

5-Hydroxytryptamine receptors (serotonin receptors):

	Nomenclature	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}
	agonists	8-OH-DPAT	CP93129	LY694247
5	antagonists	WAY100135(7.2-7.7)		GR127935(8.5)

	Nomenclature	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	5-HT ₃	5-HT ₄
	agonists	α -methyl-5-HT	α -methyl-5-HT	α -methyl-5-HT	2-methyl-5-HT	renzapride
10	antagonists	ketanserin-(8.5-9.5)	SB200646-(7.5)	mesulergine(9.1)	granisetron(10)	GR113808-(9-9.5)

Leukotriene receptors:

endogenous agonists: LTB₄, LTC₄, LTD₄, LTE₄

	Nomenclature	LTB ₄	LTC ₄	LTD ₄
15	agonists	LTB ₄		
	antagonists	LY255283(7.2)	BAYu9773	ICI98615(7.9-9.3)

Muscarinic receptors:

20	Nomenclature	M ₁	M ₂	M ₃	M ₄
	agonists				
	antagonists	pirenzepine-(8.0)	methoctamine-(7.9)	hexahydrosiladifenidol(8.0)	tropicamide-(7.8)

Neuropeptide Y receptors:

endogenous agonists: neuropeptide Y, peptide YY, pancreatic polypeptide

	Nomenclature	Y_1	Y_2
5	agonists	$[\text{Pro}^{34}]\text{NPY}$	NPY_{13-36}
	antagonists		

Prostanoid receptors:

10 endogenous agonists: PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$, PGH_2 , PGI_2 , TXA_2

	Nomenclature	DP	FP	IP	TP
	agonists	BW245C	fluprostenol	cicaprost	U46619
	antagonists	BWA869C(9.3)			GR32191(8.8)

15	Nomenclature	EP1	EP2	EP3
	agonists	17-phenyl- ω -trinol- PGE_2	butaprost	sulprostone
	antagonists	SC19220(5.6)		

P₂ Purinoceptors:

endogenous agonists: AMP, ADP, ATP, UTP

	Nomenclature	P _{2X}	P _{2Y}	P _{2Z}	P _{2T}	P _{2U}
5	agonists	α,β -methylene ATP	2-methylthio ATP		2-methylthio ADP	UTP γ S
	antagonists	suramin(5.0)	suramin-(5.0)	2-methylthio-L-ATP	suramin(5.0)	

Tachykinin receptors:

10 endogenous agonists: substance P, neurokinin A, neurokinin B, neuropeptide K, neuropeptide γ

	Nomenclature	NK ₁	NK ₂	NK ₃
	agonists	Substance P methylester	GR64339	senktide
15	antagonists	SR99994	SR8968(9.0)	GR138676

Vasopressin receptors:

endogenous agonists: vasopressin, oxytocin

	Nomenclature	V ₁	V ₂
20	agonists		d[DAArg ⁸]VP
	antagonists	SR49059(9.4)	OPC31269

Calcitonin gene-related peptide receptors:

	Nomenclature	CGRP rec.	Amylin rec.
	agonists		
5	antagonists		Amylin ₈₋₃₇

Ca²⁺ channels:

	Channel type	L
10	blocker	verapamil nifedipine

Nitric oxide receptors:

	Nomenclature	soluble guanylyl cyclase
15	agonists	nitroglycerin Na-nitroprusside
	antagonists	L-NMMA

Other ligand-receptor systems related to the regulation of the EC-SOD synthesis:**Platelet derived growth factor:**

20	endogenous agonists: PDGF-AA, PDGF AB, PDGF BB receptors: PDGFR- α , PDGFR- β antagonists: neomysin: trapidil, neutralising antibodies, soluble receptor fragments
----	---

Fibroblast growth factor:

endogenous agonists: acidic FGF, basic FGF

receptors: FGFR-1, FGFR-2, FGFR-3, FGFR-4

- 5 antagonists: neutralising antibodies, soluble receptor fragments
-

Transforming growth factor β :

endogenous agonists: TGF β 1, TGF β 2, TGF β 3, TGF β 4

- 10 receptors: TGF β R-I, TGF β R-II, TGF β R-III

antagonists: decorin, neutralising antibodies, soluble receptor fragments

Interferon γ :

-
- 15 endogenous agonists: Interferon γ

receptors: IFN γ R

antagonists: neutralising antibodies, soluble receptor fragments

Tumor necrosis factor:

20

endogenous agonists: TNF α , TNF β

endogenous antagonists: soluble type I TNF-R, soluble type II TNF-R

receptors: TNF-RI, TNF-RII

antagonists: neutralising antibodies, soluble receptor fragments

25

Interleukin-1:

endogenous agonists: $\text{Il-1}\alpha$, $\text{Il-1}\beta$

endogenous antagonists: "endogenous Il-1 receptor antagonist"

5 receptors: Il-1-R

antagonists: neutralising antibodies, soluble receptor fragments

Interleukin-4:

10 endogenous agonists: Il-4

receptors: Il-4-R

antagonists: neutralising antibodies, soluble receptor fragments

Interleukin-8:

15

endogenous agonists: Il-8 , $\text{GRO}\alpha$, NAP-2

receptors: Il-8_A , Il-8_B

antagonists: neutralising antibodies, soluble receptor fragments

20 **Interleukin-10:**

endogenous agonists: Il-10

receptors: Il-10-R

antagonists: neutralising antibodies, soluble receptor fragments

25

Interleukin-13:

endogenous agonists: Il-13

receptors: Il-13-R

30 antagonists: neutralising antibodies, soluble receptor fragments

Growth hormone:

endogenous agonist: GH

endogenous antagonist: soluble GHR

5 receptors: GHR

antagonists: neutralising antibodies, soluble receptor fragments

Thrombin:

10 endogenous agonist: thrombin, thrombin receptor agonist

receptors:

antagonists: neutralising antibodies, soluble receptor fragments

agonists: thrombin receptor related polypeptides

Heparin receptors, receptors for other sulfated glycosaminoglycans:

15

endogenous agonists: heparin, heparan sulfate, other sulfated glycosaminoglycans, fragments of these

synthetic: low-molecular weight heparin, synthetic other negatively charged saccharides, and polymers

20 endogenous antagonists: heparitinases, heparinases

Epidermal growth factor:

endogenous agonists: EGF, HB-EGF, TGF- α

receptor antagonists: EGF-R, neutralising antibodies, soluble receptor fragments

25 **Protein kinase C:**

PKC activator: phorbol, 12-myristate, 13-acetate, synthetic activators

PKC inhibitor: staurosporine, synthetic inhibitors

Insulin:

agonist: insulin, insulin-like growth factors

receptor: insulin receptor

5

In Example 2 it is demonstrated how a potential vasoactive factor, growth factor or other factors influencing the synthesis of EC-SOD can be tested and selected. The present invention relates in particular to a substance selected from
10 the group consisting of angiotensin, bradykinin, endothelin, histamine, serotonin, thrombin, vasopressin and substances releasing nitric oxide such as Na-nitroprusside, glyceryl trinitrate, isosorbide dinitrate, isosorbide mononitrate and SIN-1. Also, the invention relates to substances related to
15 inflammation, such as a substance selected from the group consisting of IFN γ , IL-4, IL-8 and indomethacin. Further, the invention relates to the use according to the invention wherein the substance is a growth factor such as growth hormone or insulin. Moreover, the invention relates to the
20 use according to the invention wherein the substance is heparin, or other sulfated glycosaminoglycans or fragments thereof, or phorbol 12-myristate 13-acetate or other factors stimulating protein kinases C.

It is well within the skill of the man skilled in the art to
25 test other substances exhibiting agonist activity against one or more of the receptors listed above - e.g. a substance already listed as an agonist - and to judge whether the substance is capable of stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD from cells
30 by use of the experimental protocol outlined in Example 2. Also other natural or synthetic factors interacting with other cellular mechanisms can be screened with regard to ability to influence EC-SOD synthesis. Generally, a 2 fold or larger, such as more than 3 fold or 6 fold, change is preferable.
35

A further aspect of the invention thus relates to a method for determining the effect of a substance with respect to stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells comprising

- 5 (a) growing human cells capable of growing in culture and releasing or synthesizing EC-SOD in an appropriate medium,
- (b) administering the substance to the medium, optionally collecting and replacing the medium containing the
10 active substance at appropriate intervals such as every 24 hours,
- (c) collecting the medium or media if replaced during the experiment and/or the supernatant of the washed and homogenized cells used in the experiment, and
- 15 (d) determining the effect of the substance by determining the amount of human EC-SOD protein in cell culture media and/or cell homogenates, and/or assessing the effect of the substance by determining the amount of human EC-SOD protein in the cell homogenate, and/or determining the content of mRNA encoding
20 EC-SOD in the cell homogenates.

Within the scope of the invention is substances which have been selected by the method outlined above. A further aspect of the invention is use of a substance, the effect of which
25 with respect to stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells has been established using the above method for the preparation of a pharmaceutical composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in
30 cells. Moreover, the invention relates to a method for preventing, diminishing, controlling or inhibiting a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from

the superoxide radical comprising administering, to a patient in need thereof, an effective amount of a substance the effect of which for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells has
5 been established using the above method.

It should be emphasized that the basic concept of the invention is to alter the EC-SOD content. Within the concept of this invention is thus also a method for determining the effect of a substance with respect to decreasing the level of
10 EC-SOD in blood vessels, bronchi, lung, kidney, skin, gut, uterus, cornea, joints, central nervous system, possibly other organs such as the heart by decreasing the endogenous synthesis of EC-SOD, using a test as outlined above, as well as substances selected by a such method, use of a such substance and a method for preventing, diminishing, controlling
15 or inhibiting a disease or disorder using a substance selected by a method as outlined above and which decreases the level of EC-SOD.

The agonist or antagonist may be prepared as formulations in
20 pharmaceutically acceptable media, for example, saline, phosphate buffered saline (PBS), Ringer's solution, dextrose/saline, Hank's solution, and glucose. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as
25 buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like. Additives may also include additional active ingredients, e.g. bactericidal agents, or stabilizers. The amount administered to the patient will vary depending upon what is being administered, the purpose of the
30 administration, such as prophylaxis or therapy, the state of the host, the manner of administration, and the like.

The pharmaceutical compositions are typically intended for oral, transdermal or parenteral administration, e.g. intravenously, subcutaneously, or intramuscularly, or for delivery
35 through inhalation, e.g. by means of a metered dose inhaler

(MDI) or a dry powder inhaler (DPI). Orally administrative forms are desirable and can, if necessary, be provided by modifying the composition to bypass the stomach environment. The composition can be used for prophylactic and/or therapeutic treatment. Alternatively, the pharmaceutical compositions can be administered intravenously. Thus, the invention provides compositions which comprise an agonist substance dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered.

The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The agonist may also be administered with a second biologically active agent.

In therapeutic applications, the pharmaceutical compositions are administered to a patient in an amount sufficient to produce the desired effect, defined as a "therapeutically effective dose". The therapeutically effective dose of an agonist will vary according to, for example, the particular use for which the treatment is made, the manner of administration, the health and condition of the patient, and the judgement of the prescribing physician. For example, the dose for continuous infusion will typically be in the range of about 500 ng to about 800 μ g per day for a 70 kg patient, preferably between about 10 μ g and about 300 μ g. The dose will typically be between 700 ng/kg/day and 16 μ g/kg/day.

The concentration of the agonist in the pharmaceutical formulations can vary widely, i.e. from about 10 μ g to about 5 mg/ml, preferably between about 100 μ g and about 2 mg/ml. The concentration will usually be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for intravenous infusion could be made up

to contain 250 ml of dextrose/saline solution and 2.5 mg of the agonist.

For solid compositions, conventional non-toxic solid carriers may be used which include, for example, pharmaceutical grades
5 of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable non-toxic composition is formed by incorporating normally employed excipients, such as those
10 carriers previously listed, and generally 10-95% of active ingredient, that is, an agonist substance, preferably 25-75%.

For aerosol administration, the agonist is preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of agonist are 0.01-20% by
15 weight, preferably 1-10%. The surfactant must, of course, be non-toxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, lino-
20 lenic, olestERIC and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arbutol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these
25 esters. Mixed esters, such as mixed or natural glycerides may be employed.

The surfactant may constitute 0.1-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquified propellants are typically
30 gases at ambient conditions, and are condensed under pressure. Among suitable liquified propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be employed. In producing the
35 aerosol, a container equipped with a suitable valve is filled

with the appropriate propellant, containing the finely divided peptide(s) and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

- 5 To enhance the serum half-life, the agonist may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional techniques may be employed which provide an extended lifetime of the peptides. Thus, in certain embodiments, the agonist may be encapsulated in a
10 liposome.

Another aspect of the present invention relates to the use of a substance for the manufacture of a composition for prophylaxis or treatment of a disease or disorder connected with the presence or formation of superoxide radicals and other
15 toxic intermediates derived from the superoxide radical, in particular to use according to the invention wherein the substance alters the level of EC-SOD in blood vessels, bronchi, lung, skin, uterus, gut, joint, cornea, kidney, central nervous system, and/or other organs such as the heart by altering
20 the endogenous synthesis of EC-SOD, e.g. wherein the disease or disorder is altered blood pressure, inflammation or formation of atherosclerotic lesions, reconstriction after arterial angioplasty, bronchial diseases involving inflammation and constriction such as asthma, other lung disorders,
25 diseases or disorder selected from conditions involving ischaemia followed by reperfusion, e.g. infarctions such as heart, kidney, brain or intestine infarctions, inflammatory diseases such as rheumatoid arthritis, pancreatitis, in particular acute pancreatitis, colitis, pyelonephritis and other
30 types of nephritis, and hepatitis, keratitis, otitis media with effusion, autoimmune diseases, diabetes mellitus, disseminated intravascular coagulation, fatty embolism, adult respiratory distress, infantile respiratory distress, brain haemorrhages in neonates, burns, wound healing, adverse
35 effects of ionizing radiation, and carcinogenesis.

Within the concept of the present invention is also the use of a substance selected from the group consisting of PDGFAA, PDGFBB, A-FGF, B-FGF, EGF and derivatives thereof for the manufacture of a composition for decreasing the release of
5 EC-SOD from cells, e.g. smooth muscle cells, or decreasing the synthesis of EC-SOD in cells, in particular in a human, such as the use of such a substance for prophylaxis or treatment of atherosclerosis or other vascular disease.

In a further aspect, the present invention relates to the use
10 of a substance selected from the group consisting of interferon- γ , prostaglandin E_2 , indomethacin, interleukin-1, TNF, TGF β and derivatives thereof for the manufacture of a composition for decreasing the release of EC-SOD from cells (e.g. smooth muscle cells) or decreasing the synthesis of EC-SOD in
15 cells, in particular in a human.

Moreover, the invention relates to a method of preventing, diminishing, controlling or inhibiting a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxi-
20 de radical in a patient who has been established to have a high risk of developing a such disease or disorder, or who has developed a such disease or disorder, the method comprising administering an effective amount of a substance which is capable of stimulating the release of EC-SOD from cells or
25 stimulating the synthesis of EC-SOD in cells.

In particular, the invention relates to a method as outlined above wherein the disease or disorder is selected from the group consisting of altered blood pressure, inflammation or formation of atherosclerotic lesions, proliferation of ar-
30 terial intima, diabetes, bronchial diseases involving inflammation and constriction such as asthma, conditions involving ischaemia followed by reperfusion, e.g. infarctions such as heart, kidney, brain or intestine infarctions, inflammatory diseases such as rheumatoid arthritis, pancreati-
35 tis, in particular acute pancreatitis, enteritis, colitis,

pyelonephritis and other types of nephritis, and hepatitis, keratitis, otitis media with effusion, autoimmune diseases, central nervous system degenerative disorders such as ALS, Parkinson's disease, Alzheimer's disease, diabetes mellitus, 5 disseminated intravascular coagulation, fatty embolism, adult respiratory distress, other lung disorders, infantile respiratory distress, brain haemorrhages in neonates, burns, adverse effects of ionizing radiation, and carcinogenesis.

10 A particular embodiment of the above is a method wherein the patient is a patient who has been established to have a high risk of developing a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical by having a high-risk-indicating score of a serum or plasma marker for 15 said disease such as high content of glycated hemoglobin (diabetes, diabetes complications), high content of lipid hydroperoxide in plasma (atherosclerosis, diabetes), altered amount of EC-SOD (such as reduced amount because of the person being e.g. heterozygous for null allele or other muta- 20 tion), high content of nitrotyrosin indicative of inflammation (with increased formation of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$), or by having a gene or gene product which indicates that the patient is at high risk of developing a disease or disorder as mentioned above.

25 It should be understood that the aspects of the invention described above may similarly be described as use of a substance for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells or methods of treatments of a patient in need of such treatment.

LEGEND TO FIGURES

Figure 1 shows immunostaining of EC-SOD in nondiseased thoracic aorta from a 40 year old man, collected 48 hours post mortem. The anti EC-SOD antibody used (1.4 $\mu\text{g/ml}$) (A), was
5 raised against the recombinant enzyme in rabbit. Nonimmunized rabbit IgG was used as a negative control (2.4 $\mu\text{g/ml}$) (B). e, endothelium; i, intima; m, media; and a, adventitia. Bar = 200 μm .

Figure 2 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
10 Example 2 were performed using endothelin 1 as an active substance on smooth muscle cells.

Figure 3 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
15 Example 2 were performed using angiotensin 2, vasopressin or endothelin 2 as an active substance on smooth muscle cells.

Figure 4 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
20 Example 2 were performed using trombin or bradykinin as an active substance on smooth muscle cells.

Figure 5 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
Example 2 were performed using histamine or serotonin as an active substance on smooth muscle cells.

25 Figure 6 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in Example 2 were performed using Na-nitroprusside, SIN-1 or growth hormone as an active substance on smooth muscle cells.

Figure 7 is a graph showing the results of the EC-SOD analysis
30 when the synthesis regulation experiments described in

Example 2 were performed using heparin or EGF as an active substance on smooth muscle cells.

Figure 8 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
5 Example 2 were performed using insulin as an active substance on smooth muscle cells.

Figure 9 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
Example 2 were performed using phorbol ester (PMA) as an
10 active substance on smooth muscle cells.

Figure 10 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
Example 2 were performed using phorbol ester (PMA) as an
active substance on fibroblasts.

15 Figure 11 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
Example 2 were performed using IFN γ as an active substance on a glia cell line.

Figure 12 is a graph showing the results of the EC-SOD analysis when the synthesis regulation experiments described in
20 Example 2 were performed using TGF β as an active substance on a malignant glioma cell line.

EXAMPLES

EXAMPLE 1

SOD-isoenzymes in human tissues and in the blood vessel wall of man and other mammals

5 Methods

Extraction of the vessel wall, bronchi and skin

Human tissues were obtained within 24 hours after death from accident victims without known disease at the Department of Forensic Medicine, Umeå University Hospital, Sweden. The
10 tissues were kept at -80°C until preparation. The tissues were homogenized with an Ultra-Turrax in 10 vol of Na acetate, pH 5.5 containing 0.3 M KBr. The homogenates were then sonicated and finally extracted for 30 minutes at 4°C . The supernatants were employed after centrifugation ($20,000 \times g$
15 for 15 minutes) for the further analyses.

Macroscopically normal pieces (0.5-1.5 g) of human left anterior descending coronary artery (LAD), proximal thoracic aorta, saphenous vein, bronchi and skin were cut out at autopsy within 48 h after death. Thoracic aortas from the other
20 mammals were collected within a few hours after death. The pieces were kept at -80°C prior to analysis. For extraction, frozen pieces were pulverized in a Braun Microdismembrator II (B Brown Biotech Inc, Allentown, PA) and the frozen powder added to 10 volumes of 50 mM potassium phosphate, pH 7.4,
25 with 0.3 M KBr, and a set of antiproteolytic agents (phenyl-methylsulfonylfluoride 0.5 mM, diethylenetriamine pentaacetic acid 3 mM, aprotinin 90 mg/l, pepstatin 10 mg/l, chymostatin 10 mg/l and leupeptin 10 mg/l). The homogenates were then sonicated and finally extracted for 30 min at 4°C . The ex-
30 tracts were then centrifuged ($20,000 g$ for 15 min). Unless analysed immediately, the supernatants were stored at -80°C .

Extraction of other human tissues

SOD activity analysis

SOD enzymatic activity was determined using the direct spectrophotometric method employing KO_2 (Marklund, 1976) as modified (Marklund, 1985). 3 mM cyanide was used to distinguish between the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the resistant Mn-SOD. One unit in the assay is defined as the activity that brings about a decay of O_2^- concentration at a rate of 0.1 s^{-1} in 3 ml of buffer. It corresponds to 8.3 ng of human CuZn-SOD, 6.3 ng of bovine CuZn-SOD, 8.6 ng of human EC-SOD and 65 ng of bovine Mn-SOD. The " KO_2 -assay" is carried out at pH 9.5 and at relatively high superoxide concentration. In comparison, the xanthine oxidase-cytochrome C SOD assay (McCord and Fridovich, 1969) is carried out under more physiological conditions, i.e. neutral pH and low superoxide concentration. One unit in the " KO_2 -assay" corresponds to about 0.024 units of CuZn-SOD and EC-SOD and 0.24 units of Mn-SOD, respectively in the "xanthine oxidase" assay. The " KO_2 -assay" is thus about 10 times more sensitive for CuZn-SOD and EC-SOD activity than Mn-SOD activity.

Specific analysis of EC-SOD

EC-SOD in human blood vessel wall, bronchi and skin extracts was determined by ELISA (Karlsson and Marklund, 1988). For conversion of results to activity units, 8.6 ng per unit was assumed (Tibell et al., 1987).

For specific analysis of EC-SOD in vessel extracts from other species and from other human tissues, chromatography on Con A-Sepharose (Pharmacia Biotech, Sollentuna, Sweden) was used. Unlike CuZn-SOD and Mn-SOD, the glycoprotein EC-SOD binds to the lectin concanavalin A. The procedure has been described previously (Marklund, 1984), the only difference being that the extraction buffer described above was used as a solvent in all steps. The yield of EC-SOD in the procedure was tested

with human blood vessel extracts. 75 % of the applied EC-SOD was found to be recovered as determined by ELISA and all EC-SOD results for the other mammals were corrected accordingly. The CuZn-SOD activity of the extracts was then calculated as total cyanide-sensitive activity minus (corrected) EC-SOD activity.

Immunohistochemistry

Vessels for immunostaining (LAD, thoracic aorta and saphenous vein) were obtained at autopsy within 48 hours of death or immediately at vessel surgery. Cryostat sections were fixed for 45 minutes in 1 % paraformaldehyde solution. An avidin-biotin-horseradish-peroxidase system (DACOPATTS, Glostrup, Denmark) was used for immunostaining. Anti-EC-SOD antibodies, raised against recombinant human protein (Tibell et al., 1987) in goat and rabbit, and purified by adsorption/desorption on EC-SOD immobilized on CNBr-activated sepharose, were used at concentrations 0.7-8.6 µg/ml. As negative controls, primary antibodies were substituted with non-immunised goat/-rabbit IgG (2.4-11.6 µg/ml). Serial sections were stained by eosin for histological orientation.

Results

SOD-isoenzymes in human tissues

CuZn-SOD, Mn-SOD and EC-SOD were determined as described in Methods above. Where ng values are given for EC-SOD, the level were determined with ELISA, in other cases with the ConA-sepharose method (Marklund, 1984). The results are outlined in Table 1.

Table 1

SOD-isoenzymes in human tissues

		CuZn-SOD	Mn-SOD	EC-SOD	EC-SOD	protein	DNA
		U/g ww	U/g ww	U/g ww	ng/g ww	mg/g ww	mg/g ww
	bronchi, n=3	3209	165	1991	17125	25	0.720
	skin, n=1	1461	65	1158	9958	11	0.510
5	uterus, n=2	7475	282	1260		22	
	brain, grey matter, n=2	21850	907	63		48.8	
	brain, white matter, n=2	11050	285	125		12.8	
10	kidney cortex, n=2	30150	289	230		114.7	
	kidney medulla, n=2	15850	1060	395		76.0	
	liver, n=2	84950	2280	68.5		84.0	
	lung, n=2	7600	371	545		91.5	
15	heart, n=2	13550	252	225		54.9	
	skeletal muscle, n=2	12800	463	74.5		70.9	
	adipose tissue, n=1	660	46	42		3.5	
	adrenal gland, n=2	23050	1260	155		38.3	
20	duodenum, n=2	8650	283	395		24.7	
	ovary, n=1	13200	147	420		47.7	
	pancreas, n=2	9965	641	650		41.4	
	spleen, n=2	13250	335	89		63.1	
	thyroid gland, n=2	12600	276	1210		79.9	

25 Levels of SOD isoenzymes in human blood vessel walls

The vessel walls were homogenized and the extracts were assayed for contents of CuZn-SOD and Mn-SOD activity, EC-SOD protein, total protein and DNA. The results for EC-SOD protein converted to activity units are also presented. The conversion factor 8.6 ng per unit was used. To obtain the CuZn-SOD activities, the cyanide-sensitive SOD activities were subtracted with these calculated EC-SOD units. Note that the

SOD activity assay used (Marklund, 1976; Marklund, 1985) is 10 times less sensitive to Mn-SOD activity than to CuZn-SOD and EC-SOD activity. The results are presented as means \pm S.D.

5

Table 2

Levels of SOD isoenzymes in human blood vessel walls

Tissue	EC-SOD		CuZn-SOD	Mn-SOD	Protein	DNA
	ng/g ww	U/g ww	U/g ww	U/g ww	mg/g ww	mg/g ww
LAD (n = 12)	30700 \pm 14400	3560 \pm 1680	4910 \pm 1880	160 \pm 62	26 \pm 8	0.81 \pm 0.14
10 Thoracic aorta (n = 18)	55400 \pm 26900	6440 \pm 3130	7040 \pm 2970	86 \pm 53	24 \pm 13	0.68 \pm 0.16
Saphenous vein (n = 10)	22300 \pm 9900	2600 \pm 1150	7000 \pm 5010	307 \pm 134	37 \pm 8	0.68 \pm 0.12

- 15 In most human tissues, CuZn-SOD is the predominant SOD isoenzyme, with Mn-SOD being about half as abundant, Table 1 and Marklund, 1984. EC-SOD normally accounts for less than 10% of the total SOD activity. Compared with other tissues, the human blood vessel wall is found to contain exceptionally
- 20 large amounts of EC-SOD which is comparable in abundance to CuZn-SOD, Table 2. Compared with other tissues there is relatively little CuZn-SOD and Mn-SOD.

SOD isoenzymes in aorta from various mammalian species

- 25 The aortas were extracted and analysed with the ConA-Sepharose procedure as described under Methods. In species in which 4 or more samples were analysed the results are presented as means \pm S.D., for the others individual results are given. ww = wet weight.

Table 3
SOD isoenzymes in aorta from various mammals

		EC-SOD U/g ww	CuZn-SOD U/g ww	Mn-SOD U/g ww	Protein mg/g ww	DNA ng/g ww
	Cow (n = 4)	4250 ± 1250	3990 ± 1290	122 ± 32	38 ± 9	0.57 ± 0.05
5	Pig (n = 8)	2660 ± 353	3020 ± 508	90 ± 16	31 ± 3.5	0.98 ± 0.19
	Dog (n = 4)	161 ± 65	6850 ± 640	135 ± 23	38 ± 4.3	0.57 ± 0.12
10	Cat (n = 6)	850 ± 340	5470 ± 2140	349 ± 123	35 ± 5	0.98 ± 0.20
	Rabbit (n = 5)	2390 ± 680	6330 ± 1210	89 ± 11	41 ± 5.9	1.06 ± 0.20
	Rat (n = 8)	90 ± 16	1650 ± 460	102 ± 51	22 ± 9.4	1.62 ± 0.26
15	Mouse (pool of eight)	3410	4010	80	30	1.72

It has previously been found that the EC-SOD content of tissues shows a considerable interspecies variation, while the contents of CuZn-SOD and Mn-SOD vary to only a minor extent (Marklund, 1984). To investigate whether these differences also occur in blood vessels, the levels of the SOD isoenzymes in aortas from 8 different mammalian species was measured (Table 3). A remarkable variation in EC-SOD content was found, whereas the differences in CuZn-SOD activity are relatively small. The Mn-SOD activity displays an intermediate variation between the species.

Localisation of EC-SOD by immunohistochemistry

The distribution of EC-SOD in human aorta, LAD and saphenous veins was studied by means of immunohistochemistry in samples from several individuals. The staining of all the samples was principally identical, and the result with a thoracic aorta specimen is presented as an example, see Fig. 1. EC-SOD is apparently evenly distributed over the wall with significant amounts observed in all layers.

EXAMPLE 2

10 Reaction of cultured cells to a variety of factors

Cell culture and regulation experiments in smooth muscle cells

Human vascular smooth muscle cell lines were initiated from uterine artery media collected at hysterectomy of women suffering from uterine myoma, using Waymouth MB 752/1 with 15% fetal calf serum (FCS), 72 µg/ml benzylpenicillin, 100 µg/ml, 2 mM glutamine and 1mM Na-pyruvate as medium. The lines were used between the 5th and the 8th passages.

20 For synthesis regulation experiments the cells were seeded into 12-well culture plates, bottom area 3.80 cm², and grown into near confluence. The effects of substances were studied with cells cultured both in 15% fetal calf serum (FCS) and 1% bovine serum albumin (BSA). For the cells studied in 1% BSA, the medium was exchanged twice to medium with 1% BSA about 20 h before the start of the experiments. The experiments were started by exchange to 0.5 ml medium with 15% FCS or 1% BSA containing indicated concentrations of active substances or only medium with 15% FCS or 1% BSA (controls). Every 24 hours the media were collected and replaced with fresh media containing active substances. At the end of the experiments, after 4 days, the media were collected and the wells were

washed 3 times with 0.15 M NaCl. To collect and homogenize the cells, 0.5 ml of ice-cold 50 mM Na phosphate, pH 7.4, containing 0.3 M KBr, 10 mM diethylenetriamine pentaacetic acid, 0.5 mM phenylmethylsulfonyl fluoride and 100 KIU/ml
5 aprotinin (the latter three additions to inhibit proteases) was added to the wells. After sonication in the wells, with the plate bathing in icewater, the homogenates were centrifuged (20000 g x 10 min) and the supernatants were collected for analysis. All samples were kept at -80°C before assay.

10 **Cell culture and regulation experiments in fibroblasts, glia and malignant glioma cell lines**

Human skin fibroblast lines were initiated from skin punch biopsy specimens obtained from healthy volunteers. Cells were grown using Ham's F10 with 10% fetal calf serum, 100 U/ml
15 penicillin, 100 µg/ml streptomycin, 25 mM HEPES and 2 mM glutamine as medium. The lines were used between the 10th and the 20th passages.

For synthesis regulation experiments, the cells were mostly seeded into 12-well culture plates, bottom area 3.80 cm², and
20 grown into near confluence. To suppress growth and reduce interference from serum factors, the medium was exchanged twice to medium with 0.5% fetal calf serum about 20 hours before the start of the experiments. The experiments were started by exchange to 0.5 ml medium with 0.5% fetal calf
25 serum containing indicated concentrations of active factors or only medium with 0.5% fetal calf serum (controls). Every 24 hours the media were collected and replaced with fresh media containing active factors. At the end of the experiments, mostly after 4 days, the media were collected and the
30 wells were washed 3 times with 0.15 M NaCl. To collect and homogenize the cells, 0.5 ml of ice-cold 50 mM Na-phosphate, pH 7.4, containing 0.3 M KBr, 10 mM diethylenetriamine pentaacetic acid, 0.5 mM phenylmethylsulfonyl fluoride and
100 KIU/ml aprotinin (the latter three additions to inhibit
35 proteases) were added to the wells. After sonication in the

wells, with the plate bathing in ice water, the homogenates were centrifuged (20,000 x g for 10 minutes) and the supernatants were collected for analysis. All samples were kept at -80°C before assay.

5 SOD analysis

EC-SOD protein was determined in cell culture media and cell homogenates with a double antibody sandwich ELISA. Microtiter plates (Nunc; Roskilde; Denmark) were coated with 100 µl/well of a solution containing 16 µg/ml of polyclonal rabbit anti-human EC-SOD IgG antibodies (raised using purified recombinant EC-SOD as antigen) in 50 mM Na₂CO₃, pH 9.6. After 2h incubation, the wells were washed and then blocked overnight with 300 µl of blocking buffer (10mM Na phosphate, pH 7.4, 140 mM NaCl, 0.1% wt/vol Tween 20, and 0.5% BSA). For analysis, 50 µl samples, diluted if necessary with blocking buffer, were added to each well and incubated for 2h. The wells were then washed with blocking buffer, whereupon 50 µl of 3 µg/ml monoclonal anti-human EC-SOD antibody B6,H6 (produced according to Example 15 in WO87/01387) dissolved in blocking buffer was added. After 2h, the wells were washed with blocking buffer followed by addition of 50 µl of peroxidase-conjugated rabbit anti-mouse IgG (DAKOPATTS, Copenhagen, Denmark) dissolved in blocking buffer. After another 2h, the wells were washed and then developed for 10 min with 100 µl of 3.7 mM O-phenylenediamine and 0.4 mM H₂O₂ in 100 mM Na citrate, pH 5.0. After addition of 100 µl of 0.5 M H₂SO₄, the absorbance at 492 nm was determined in an ELISA processor II (Hoechst Behring).

The assay was standardized with human umbilical cord EC-SOD C. The limit of sensitivity was about 0.25 ng/ml. There was no cross-reaction with the bovine EC-SOD in the fetal calf serum.

Protein and DNA analysis

For protein analysis, Coomassie Brilliant Blue G-250 was employed, standardized with human serum albumin. DNA was determined with fluorimetry as a complex with (2-[2-(4-hydroxy-phenyl)-6-benzimidazolyl-6-(1-methyl-4-piperazyl)benzimidazol
 5 3HCl (Hoechst 33258) using calf thymus DNA as standard.

Regulation of EC-SOD in smooth muscle cells

Changes in EC-SOD levels in cell culture media, as determined by ELISA, after incubation of human vascular smooth muscle
 10 cell cultures with various substances for four days. Media containing active substances were changed daily. (Directions and levels of change are presented as compared to controls on the last days media.)

Table 4

15 Substances related to:

	<u>Inflammation:</u>	Direction:	Level of change: (-fold)
	IFN γ (5000 U/ml)	+	4*
	IL-4 (15 ng/ml)	+	2.5*
	IL-8 (1 μ g/ml)	+	2*
20	TNF α (30 ng/ml)	-	1/3
	TGF β (5 ng/ml)	-	1/6
	Il-1 α	+/-	variable
	Prostaglandin E ₂	+/-	variable
	Indomethacin (1 μ M)	+	2*

Vasoactive substances:

	Endothelin 1 (1 μ M)	+	3*
	Endothelin 2 (1 μ M)	+	3*
	Angiotensin 2 (500 nM)	+	3*
5	Vasopressin (50 nM)	+	6*
	Thrombin (10 U/ml)	+	4*
	Bradykinin (8 μ M)	+	2*
	Serotonin (10 μ M)	+	4*
	Histamine (10 μ M)	+	5*

10 NO-related:

	Na-nitroprusside (300 μ M)	+	3*
	SIN-1 (300 μ M)	+	3*

Growth factors:

	PDGF-AA (10 ng/ml)	-	1/2
15	PDGF-BB (50 ng/ml)	-	1/2
	acid FGF (120 ng/ml)	-	1/2
	basic FGF (120 ng/ml)	-	1/2
	Growth hormone (100 ng/ml)	+	3*
	EGF (20 ng/ml)	-	1/2
20	insulin (1 IU/ml)	+	2*

Other factors:

	Heparin (100 IU/ml)	+	2*
	Phorbol 12-myristate 13-acetate (3 nM)	+	3*

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CLAIMS

1. The use of a substance for the manufacture of a composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells.
- 5 2. The use according to claim 1 wherein the substance exhibits agonist activity on a receptor selected from the group consisting of adenosin receptors, adrenoceptors, angiotensin receptors, atrial natriuretic peptide receptors, bradykinin receptors, calcitonin gene-related peptide receptors, Ca^{++}
10 channels, dopamine receptors, endothelin receptors, fibroblast growth factor, growth hormone, histamine receptors, 5-hydroxytryptamine receptors, interferon γ , interleukin-1, interleukin-4, interleukin-8, interleukin-10, interleukin-13, leukotriene receptors, muscarinic receptors, neuropeptide Y
15 receptors, nitric oxide receptors, platelet derived growth factor, prostanoid receptors, P_2 purinoceptors, 5-hydroxytryptamine receptors, tachykinin receptors, thrombin receptors, transforming growth factor β , tumor necrosis factor, and vasopressin receptors, receptors for heparin and other
20 sulfated glycosaminoglycans, insulin receptors, epidermal growth factor receptors and protein kinase C.
3. The use according to claim 1 or 2 wherein the substance is a vasoactive factor.
4. The use according to claim 3 wherein the substance is
25 selected from the group consisting of angiotensin, bradykinin, endothelin, histamin, serotonin, trombin, vasopressin and substances releasing nitric oxide.
5. The use according to claim 1 or 2 wherein the substance is a substance related to inflammation.
- 30 6. The use according to claim 5 wherein the substance is selected from the group consisting of $\text{IFN}\gamma$, IL-4, IL-8 and indomethacin.

7. The use according to claim 1 or 2 wherein the substance is a growth factor.
8. The use according to claim 7 wherein the substance is growth hormone or insulin.
- 5 9. The use according to claim 1 or 2 wherein the substance is heparin, or other sulfated glycosaminoglycans or fragments thereof.
- 10 10. The use according to claim 1 or 2 wherein the substance is phorbol 12-myristate 13-acetate or other factors stimulating protein kinases C.
- 15 11. The use of a substance for the manufacture of a composition for prophylaxis or treatment of a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical.
- 20 12. The use according to claim 11 wherein the substance alters the level of EC-SOD in blood vessels, bronchi, lung, kidney, gut, central nervous system, cornea, joint, middle ear, skin, uterus, and/or other organs such as the heart by altering the endogenous synthesis of EC-SOD.
- 25 13. The use according to claim 12 wherein the disease or disorder is selected from the group consisting of altered blood pressure, inflammation or formation of atherosclerotic lesions, proliferation of arterial intima and diabetes.
- 30 14. The use according to claim 12 wherein the disease or disorder is bronchial diseases involving inflammation and constriction such as asthma.
15. The use of a substance according to claim 12 wherein the disease or disorder is selected from conditions involving ischaemia followed by reperfusion, e.g. infarctions such as

heart, kidney, brain or intestine infarctions, inflammatory diseases such as rheumatoid arthritis, pancreatitis, in particular acute pancreatitis, enteritis, colitis, pyelonephritis and other types of nephritis, and hepatitis, keratitis, otitis media with effusion, autoimmune diseases, central nervous system degenerative disorders such as ALS, Parkinson's disease, Alzheimer's disease, diabetes mellitus, disseminated intravascular coagulation, fatty embolism, adult respiratory distress, other lung disorders, infantile respiratory distress, brain haemorrhages in neonates, burns, adverse effects of ionizing radiation, and carcinogenesis.

16. A method for determining the effect of a substance with respect to stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells comprising

- 15 (a) growing human cells capable of growing in culture and releasing or synthesizing EC-SOD in an appropriate medium,
- (b) administering the substance to the medium, optionally collecting and replacing the medium containing the
20 active substance at appropriate intervals such as every 24 hours,
- (c) collecting the medium or media if replaced during the experiment and/or the supernatant of the washed and homogenized cells used in the experiment, and
- 25 (d) determining the effect of the substance by determining the amount of human EC-SOD protein in cell culture media and/or cell homogenates, and/or assessing the effect of the substance by determining the amount of human EC-SOD protein in the cell homogenate and/or determining the
30 content of mRNA encoding EC-SOD in the cell homogenates.

17. A substance which has been selected by the method according to claim 16.

18. The use of a substance, the effect of which with respect to stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells has been established using the method according to claim 16 for the preparation of
5 a pharmaceutical composition for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells.

19. A method for preventing, diminishing, controlling or inhibiting a disease or disorder connected with the presence or
10 formation of superoxide radicals and other toxic intermediates derived from the superoxide radical comprising administering, to a patient in need thereof, an effective amount of a substance the effect of which for stimulating the release of EC-SOD from cells or stimulating the synthesis of EC-SOD
15 in cells has been established using the method according to claim 16.

20. A method of preventing, diminishing, controlling or inhibiting a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical in a patient who has
20 been established to have a high risk of developing a such disease or disorder, or who has developed a such disease or disorder, the method comprising administering an effective amount of a substance which is capable of stimulating the
25 release of EC-SOD from cells or stimulating the synthesis of EC-SOD in cells.

21. A method according to claim 19 or 20 wherein the disease or disorder is selected from the group consisting of altered blood pressure, inflammation or formation of atherosclerotic lesions, proliferation of arterial intima, diabetes, bronchial diseases involving inflammation and constriction such as asthma, conditions involving ischaemia followed by reperfusion, e.g. infarctions such as heart, kidney, brain or intestine infarctions, inflammatory diseases such as
30 rheumatoid arthritis, pancreatitis, in particular acute

- pancreatitis, enteritis, colitis, pyelonephritis and other types of nephritis, and hepatitis, keratitis, otitis media with effusion, autoimmune diseases, central nervous system degenerative disorders such as ALS, Parkinson's disease,
- 5 Alzheimer's disease, diabetes mellitus, disseminated intravascular coagulation, fatty embolism, adult respiratory distress, other lung disorders, infantile respiratory distress, brain haemorrhages in neonates, burns, adverse effects of ionizing radiation, and carcinogenesis.
- 10 22. A method according claim 21 or 22, wherein the patient is a patient who has been established to have a high risk of developing a disease or disorder connected with the presence or formation of superoxide radicals and other toxic intermediates derived from the superoxide radical by having a
- 15 high-risk-indicating score of a serum or plasma marker for said disease such as high content of glycated hemoglobin, high content of lipid hydroperoxide in plasma, altered amount of EC-SOD, high content of nitrotyrosine, or by having a gene or gene product which indicates that the patient is at high
- 20 risk of developing a disease or disorder as mentioned in claim 20.

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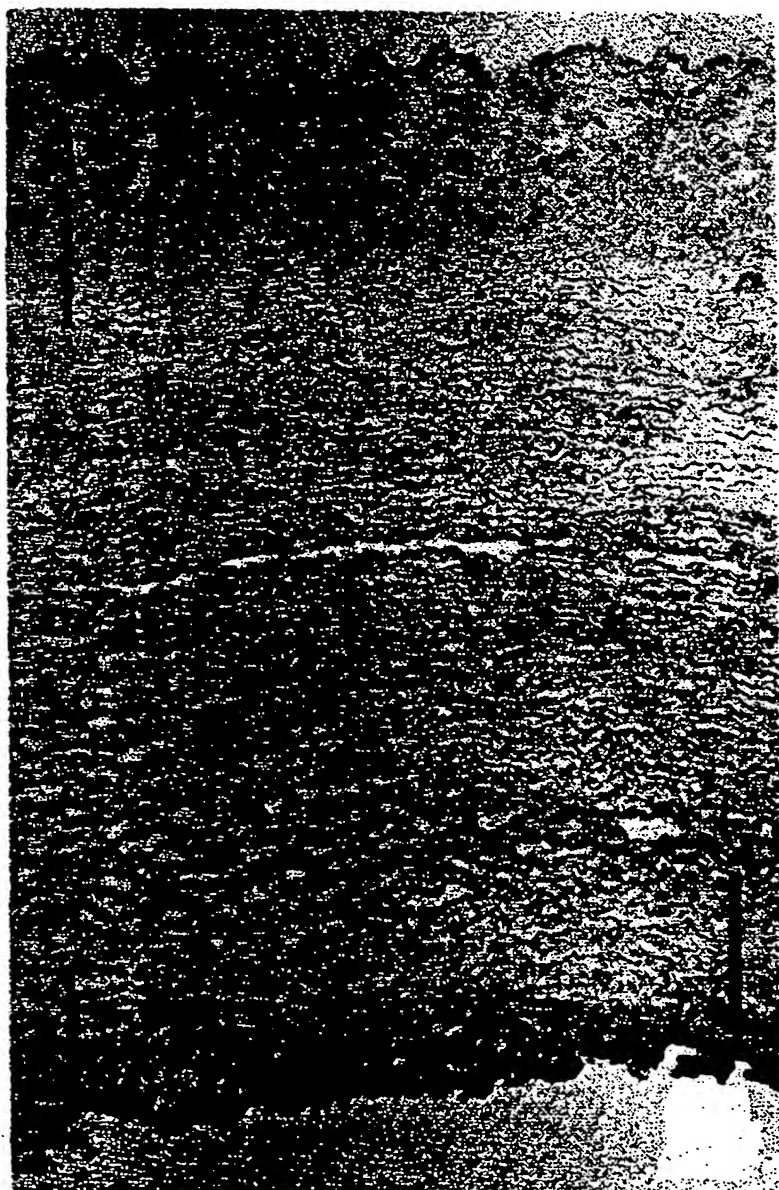


Fig. 1A

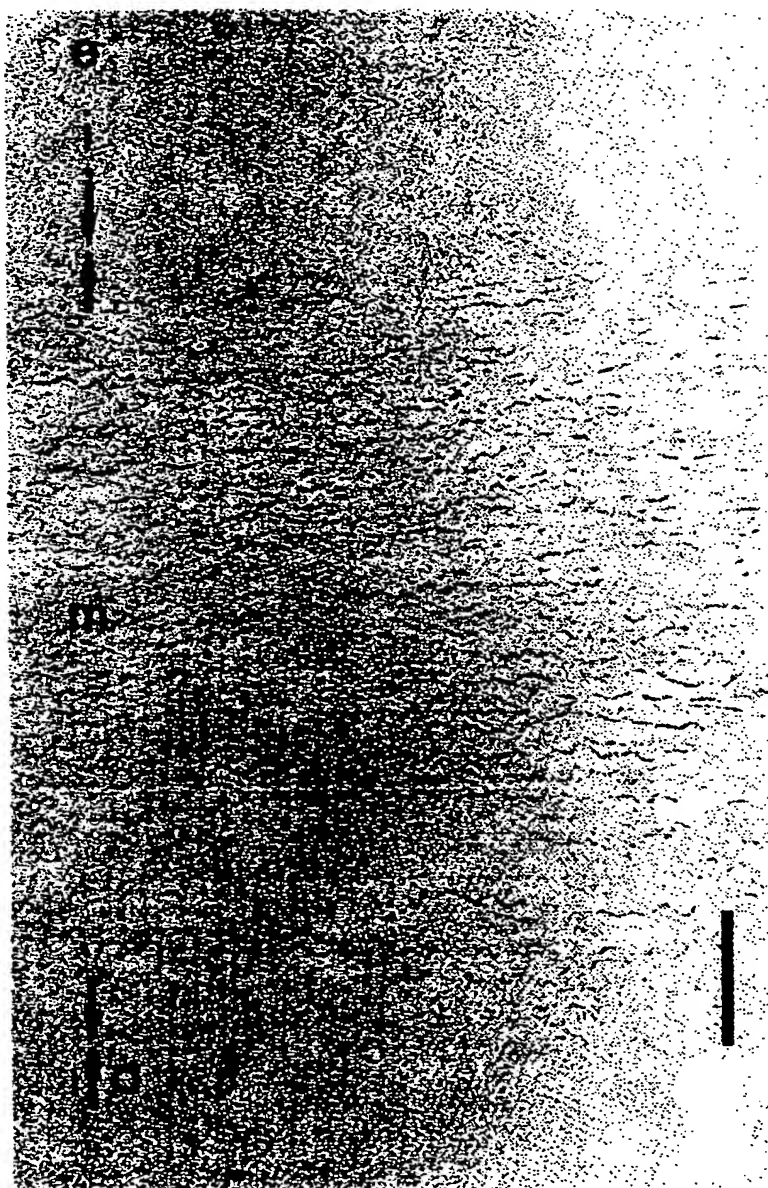


Fig. 1B

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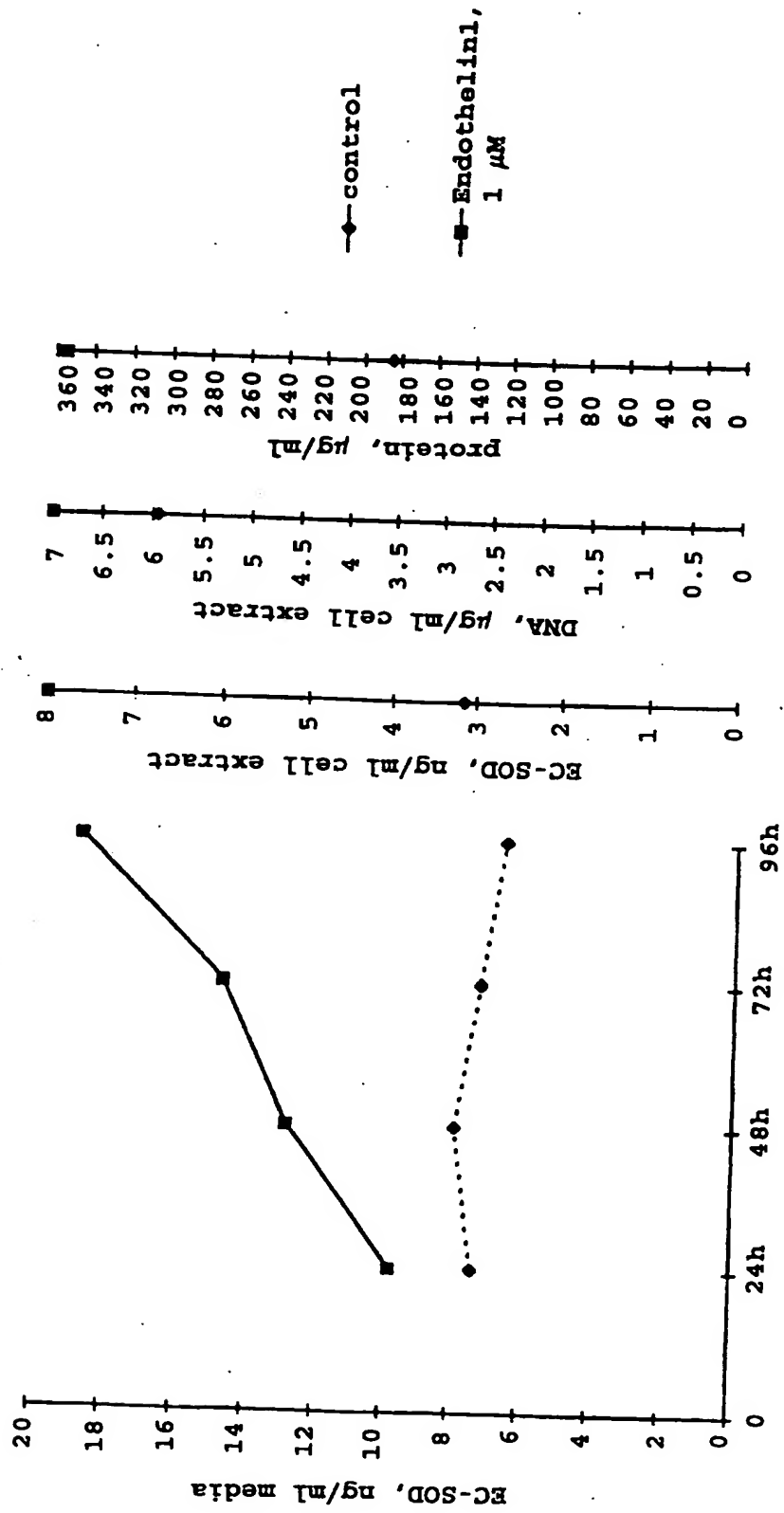


Fig. 2

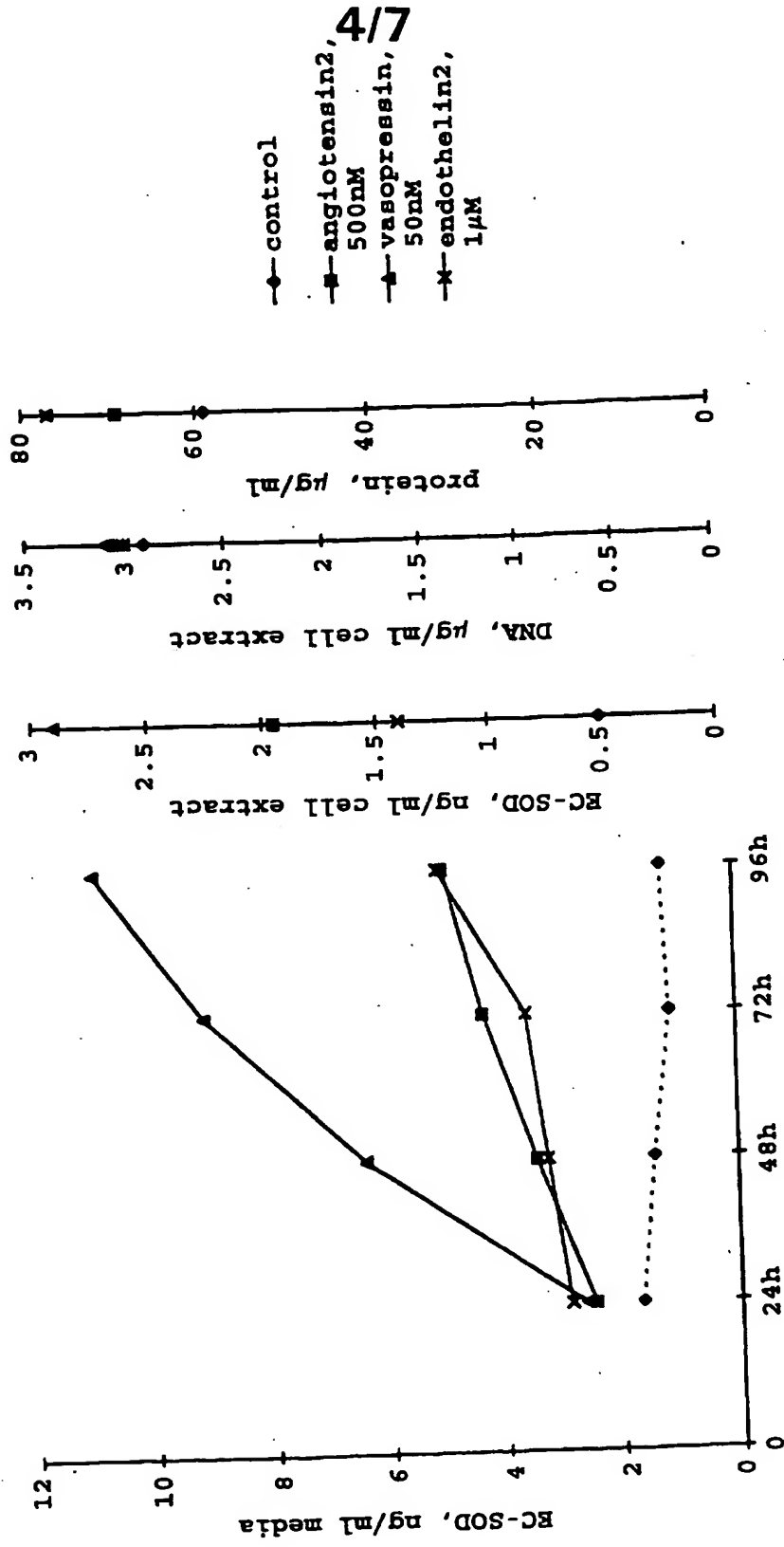


Fig. 3

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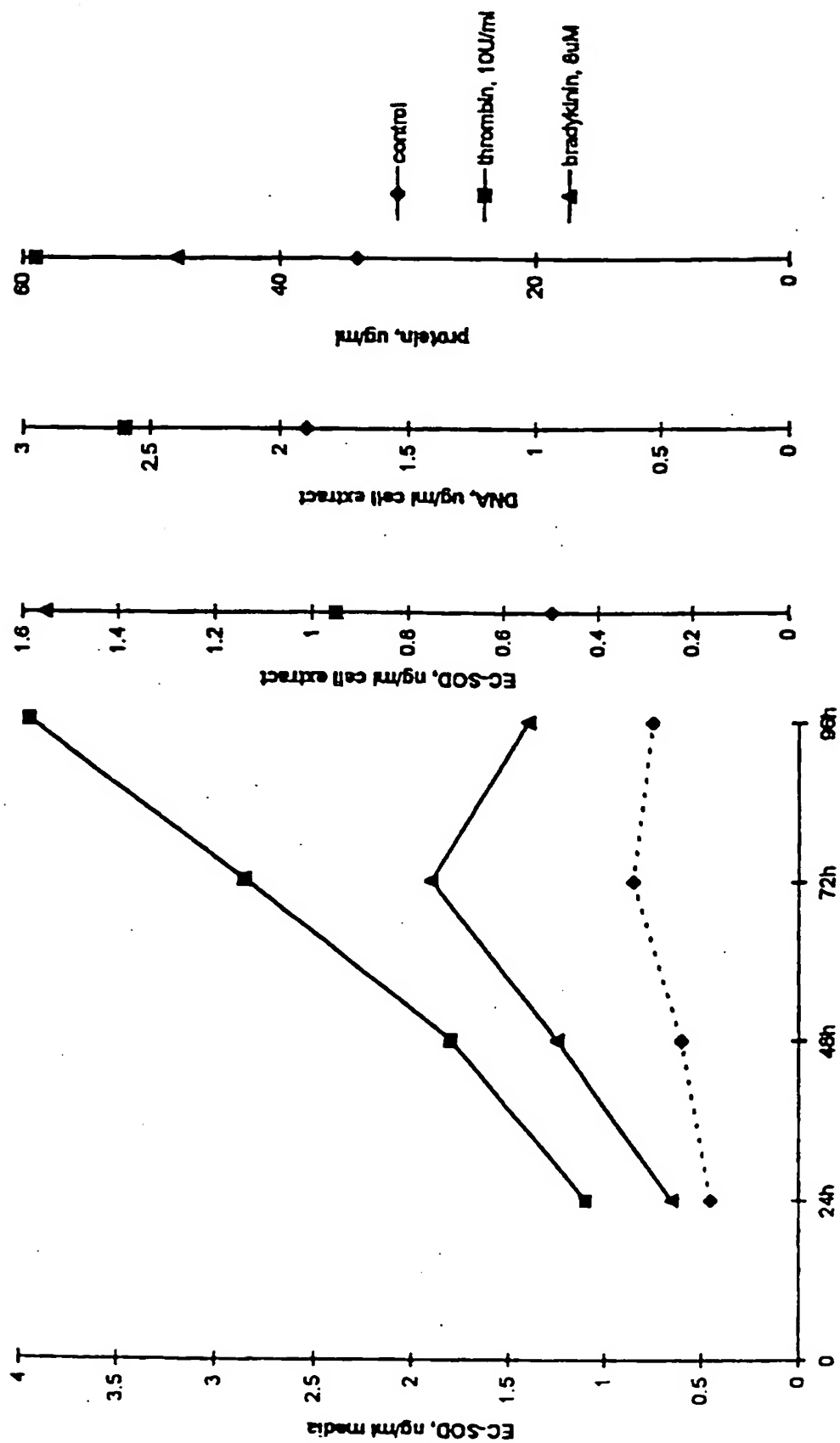


Fig. 4

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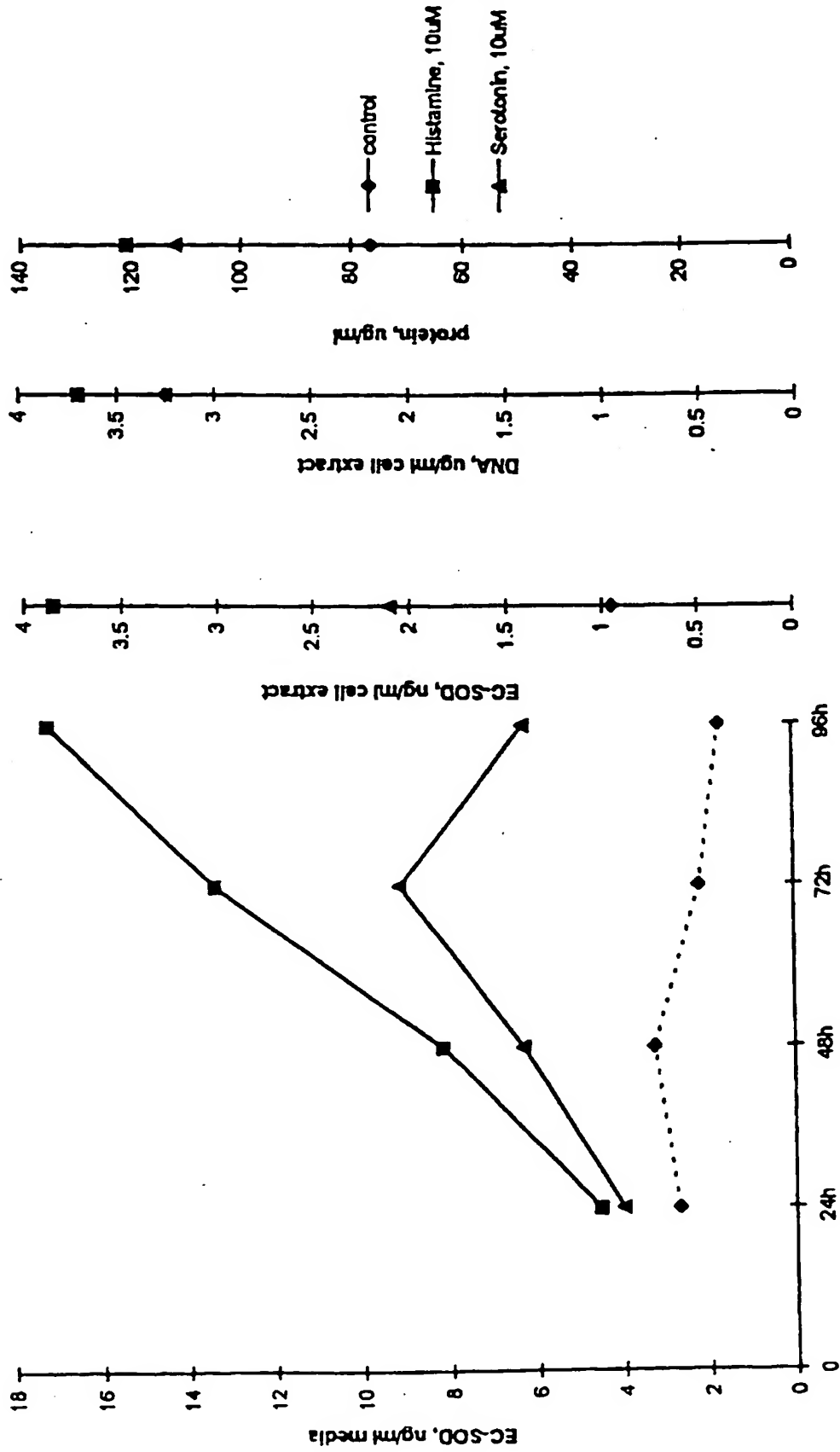


Fig. 5

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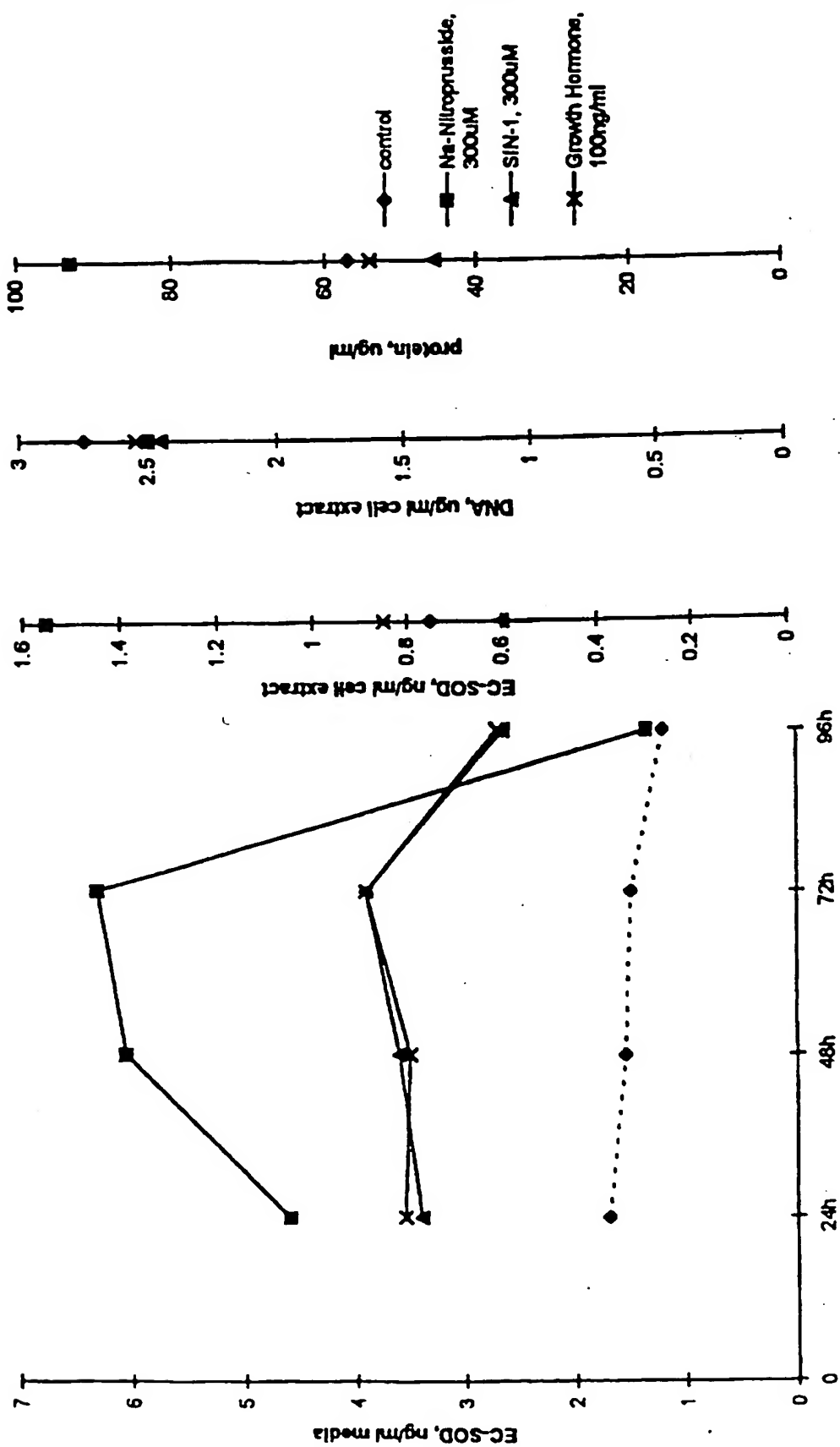


Fig. 6

INTERNATIONAL SEARCH REPORT

In ional Application No
PLI/IB 95/00979

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARTERIOSCLEROS. THROMBOS., vol. 12, no. 7, 1992 pages 824-829, H.A. LEHR ET AL. 'Superoxide-dependent stimulation of leukocyte adhesion by oxidatively modified LDL in vivo.' * discussion *	1-3,9,11
X	J. BIOL. CHEM., vol. 267, no. 10, 1992 pages 6696-6701, S.L. MARKLUND 'Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts.' see the whole document	1,2,5,6, 11-13, 16-21

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

12 February 1996

Date of mailing of the international search report

23.02.96

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